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CURE OF MURINE LEISHMANIASIS WITH ANTI-INTERLEUKIN 4 MONOCLONAL ANTIBODY

Evidence for a T Cell-dependent, Interferon γ-independent Mechanism

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Infection of inbred mouse strains with *Leishmania major* causes local infection with subsequent resolution and establishment of immunity in genetically resistant mice, but progressive, disseminated fatal disease in genetically susceptible mice. Healing in resistant strains, such as C57BL/6, is associated with the capacity of lymphocytes to generate IFN-γ in response to *Leishmania* antigens in vitro (1) and the appearance of IFN-γ mRNA in lymphoid tissues in vivo (2). IFN-γ has been identified as the major macrophage-activating factor produced by stimulated T cells (3–5), and is capable of activating macrophages to kill the obligate intracellular amastigote form of the parasite (6–8).

Susceptible BALB/c mice can be immunologically manipulated before infection, e.g., by sublethal irradiation (9) or transient depletion of $CD4^+$ T cells with the anti-CD4 mAb GK1.5 (GK1.5-BALB/c; 1, 10), such that these animals contain infection and establish protective immunity against reinfection. The control of infection in these mice has also been correlated with production of IFN-γ by T cells in vivo and in vitro (1, 2). Untreated BALB/c mice, however, do not generate significant IFN-γ, despite the demonstrable expansion of the $CD4^+$ T cell population during progressive infection (2, 11). In contrast to the healing mice, these animals have mRNA for IL-4 in lymphoid tissues in vivo together with elevated levels of serum IgE (2). These data suggest that healing or fatally infected BALB/c mice expand distinct $CD4^+$ T cell subsets that correlate with control or progression of disease. These in vivo cells would be correlates of the Th1 and Th2 cells previously distinguished and defined at the clonal level in vitro (12, 13).

Based on these observations, we administered an IL-4-specific neutralizing mAb in order to interfere with expansion and function of Th2 cell populations in infected BALB/c mice. In preliminary experiments, such therapy clearly attenuated the course of early disease (2). In experiments reported here, anti-IL-4 therapy initiated at the time of infection attenuated disease in 100% of susceptible BALB/c mice and resulted in complete resolution of disease in 85% of mice with the establishment of protective

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immunity. Further, cure was independent of IFN-γ, as judged by the failure of coadministration of neutralizing anti-IFN-γ mAb to alter the outcome of anti-IL-4 therapy. This was in contrast to resistant C57BL/6 or GK1.5-BALB/c mice, both of which developed progressive infection when treated with anti-IFN-γ mAb.

Materials and Methods

Mice. Female BALB/c and C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory, Bar Harbor, ME. Female athymic nude BALB/c (*nu/nu*) mice, 6–8 wk old, were purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN, and housed in the specific pathogen-free animal care facility at U.C.S.F. Medical Center.

Parasites. L. major (World Health Organization strain WHOM/IR/-/173) was cultured as promastigotes in M199 (Cell Culture Facility, U.C.S.F. Medical Center) containing 30% FCS (Gemini Bio-Products, Calabasas, CA) as described (14, 15). Metacyclic stage promastigotes were harvested from stationary phase cultures by centrifugation and washed in HBSS (Cell Culture Facility, U.C.S.F. Medical Center) before use.

mAbs. mAbs included GK1.5 (rat IgG2b), which recognizes the murine CD4 antigen (16), 53-6.72 (rat IgG2a), which recognizes the murine CD8 antigen (17), R4 6A2 (rat IgG1), which neutralizes murine IFN-γ (18), and 11B11 (rat IgG1), which neutralizes murine IL-4 (19).

mAbs were prepared from rat/mouse heterohybridomas grown as ascites in pristane-primed BALB/c *nu/nu* mice. Preparations were precipated in 45% ammonium sulfate, dialyzed against PBS, pH 7.2, and quantitated for protein before use (20).

IFN-γ. Purified murine rIFN-γ (lot no. 2271-54-F1; sp act, 5×10^6 antiviral U/mg; endotoxin, 0.032 E.U./mg), provided as a 1 mg/ml solution in sterile buffered saline (Genentech, Inc., South San Francisco, CA), was diluted to 100 μg/ml in sterile normal saline, aliquoted, and stored at 4°C before use. Beginning with the day of infection, designated BALB/c mice received 20 μg rIFN-γ intraperitoneally every other day for 6 wk.

Infection of Mice with mAbs. Selected BALB/c mice were injected intraperitoneally with 1.0 mg purified GK1.5 in 250 μl PBS 24 h before infection, as described (1, 10). Beginning on the day of infection, designated mice received 0.5 mg purified 11B11 or R4 6A2, or both, intraperitoneally once per week for 6 wk.

Infection of Mice. Mice were infected in the hind footpads with 4×10^6 metacyclic stage *L. major* promastigotes, as described (1, 21). Footpad swelling was determined weekly using a metric caliper. At the times designated, sera were collected and the animals were killed. The tissue from the dorsal portion of the footpads was removed intact and frozen in OCT compound (Miles Laboratories Inc., Naperville, IL). After thin sectioning on a cryostat (International Equipment Co., Needham Heights, MA), the largest transverse diameter was prepared for histologic examination after staining with modified Wright/Geimsa (Dif Quik; American Scientific Products, McGaw Park, IL). The spleens and draining (popliteal) lymph nodes were used for the isolation of mRNA (see below).

Adoptive Transfer of Cellular Immunity. Spleen and popliteal lymph node cells were isolated from anti-IL-4-healed *L. major*-infected BALB/c mice at 12–15 wk after infection. The cells were pooled and divided into three aliquots. Two were depleted of $CD4^+$ cells or $CD8^+$ cells by incubation with 10 μg/ml anti-CD4 mAb or 10 μg/ml anti-CD8 mAb, respectively, followed by incubation with 1:10 diluted complement (Rabbit Low Tox M; Accurate Chemical & Scientific Corp., Westbury, NY). Two cycles of depletion were performed with the respective antibodies. Depletion was confirmed by flow cytometry. The third group of cells received no treatment. Groups of five recipient BALB/c mice were irradiated with 200 rad (22) before intravenous administration of 10^7 cells from one of the three cell preparations. Control BALB/c mice were similarly irradiated but received 10^7 naive BALB/c spleen cells. Additional groups included untreated mice and irradiated mice that did not receive any cell transfer. All mice were infected 24 h after cell transfer. Disease progression was assessed by footpad swelling and ulceration.

Northern Blot Analyses. Harvested tissues were homogenized in 6 M guanidine hydrochloride and isolated by the method of Chirgwin et al. (23). Poly(A)$^+$ RNA was subsequently purified using oligo-dT affinity chromatography (24), subjected to electrophoresis in 6% form-

aldehyde/1% agarose gel (25), and transferred to nylon membranes by capillary blotting (Amersham Corp., Arlington Heights, IL) (26). The membranes were exposed to short-wave UV light for 30 s to fix the RNA to the nylon matrix, and treated at 42°C for 4 h with prehybridization solution containing 10% dextran before incubation with ^{32}P-labeled nucleic acid probe. The probes used were IL-4 cDNA subcloned into pSP65 (2), and the 550-bp Eco RI/Eco RV fragment of murine IFN-γ (Dr. P. W. Gray, Genentech, Inc.) subcloned into pSP72 (Promega Biotec, Madison, WI). IFN-γ and IL-4 antisense probes were generated using ^{32}P-CTP (800 Ci/mmole; New England Nuclear, Boston, MA) and SP6 RNA polymerase (Promega Biotec) as described (2). Membranes were hybridized with antisense RNA probes at 65°C overnight, and washed in 0.1% SDS, 0.1× SSC (0.015 M NaCl, 0.0015 M sodium citrate buffer, pH 7.0) at 65°C. Membranes were hybridized with nick-translated cDNA probe at 42°C overnight and washed in 0.1% SDS, 0.1× SSC at 55°C. Washed membranes were exposed to X-ray film as previously described (2).

Serum IgE Determinations. Sera isolated before death were quantitated for IgE using a sandwich ELISA with monoclonal anti-ε (EM-95) as the primary antibody and biotinylated polyclonal rabbit-anti-ε as the secondary antibody, as described (27).

Results

Effect of Exogenous rIFN-γ in Infected BALB/c Mice. The low levels of IFN-γ produced during progressive *L. major* infection in susceptible BALB/c mice, in contrast to the readily detectable levels produced by resistant C57BL/6 mice (1, 2), suggested that parenterally administered rIFN-γ might favorably influence the outcome of infection in the BALB/c mice. Based on preliminary experiments, a dose of IFN-γ that was twofold below that resulting in toxicity to infected mice was used. This dose, 20 μg every 48 h, was twice that used to cure otherwise fatal murine *Toxoplasma gondii* (28, 29) or *Trypanosoma cruzi* (30) infection. Infected BALB/c mice receiving rIFN-γ demonstrated a slight delay in footpad swelling (Fig. 1), but ulceration and histologic evidence of extensive parasitization occurred in all animals (Fig. 2 *C*). At 5 wk post-infection, however, treated mice had significant diminution in serum IgE levels compared with control-infected mice (Fig. 3), indicating in vivo efficacy of the exogenous IFN-γ (27).

Effect of In Vivo Neutralization of IL-4 on the Course of Leishmaniasis in BALB/c Mice. BALB/c mice that received six weekly injections of anti-IL-4 mAb (11B11) exhibited

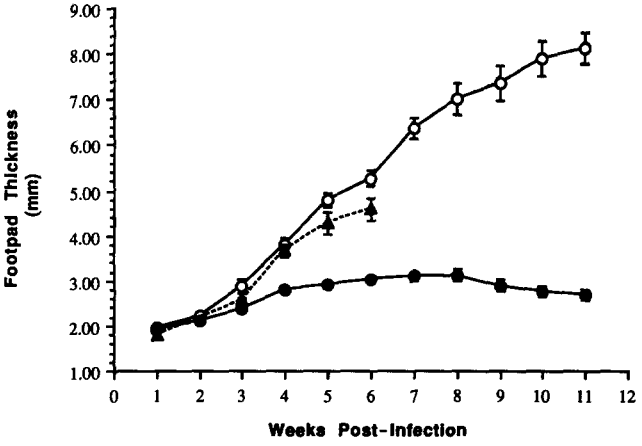

FIGURE 1. Effect of rIFN-γ or anti-IL-4 treatment on the progression of *L. major* infection in BALB/c mice. Footpad measurements represent the mean ± SEM from 48 mice after no treatment (○) or anti-IL-4 treatment (●), and the mean ± SEM from five mice after rIFN-γ treatment (▲).

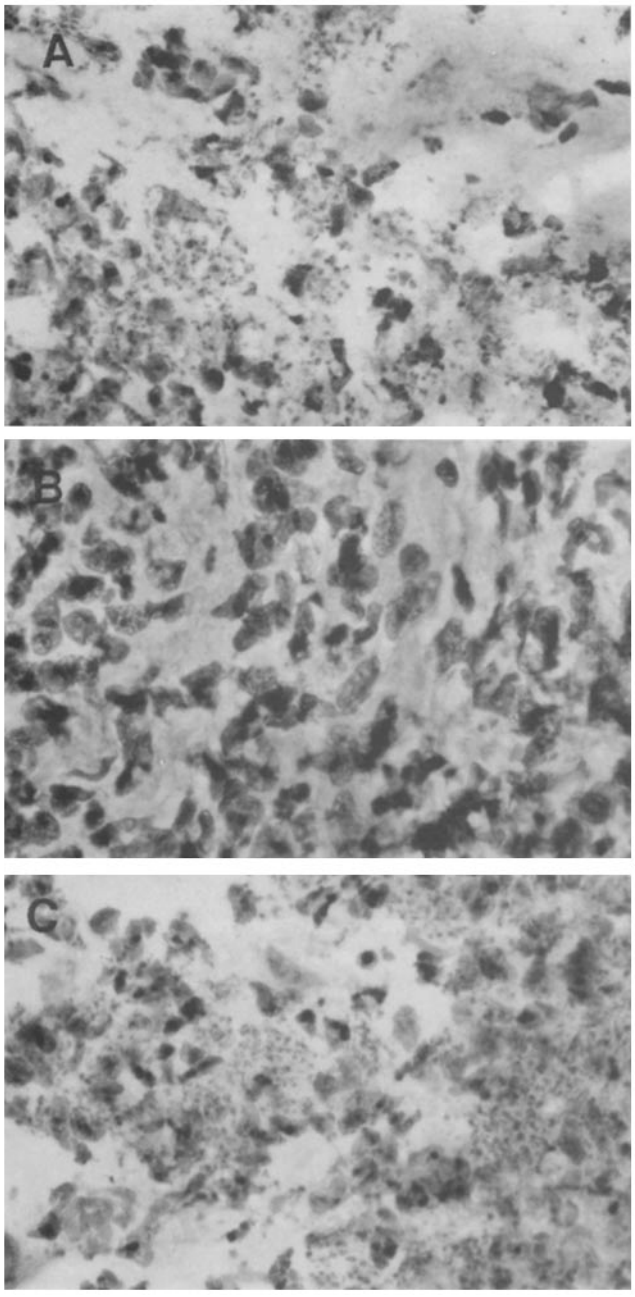

FIGURE 2. Effect of rIFN-γ or anti-IL-4 treatment on the progression of *L. major* infection in BALB/c mice. Tissue sections were prepared from the footpads of 8-wk-infected untreated (*A*), anti-IL-4-treated (*B*), and rIFN-γ- treated (*C*) BALB/c mice and represent the largest transverse diameter from the dorsal surface of the infected footpads (×750).

significant attenuation of disease as compared with matched untreated animals (Fig. 1). Footpad swelling was markedly decreased and began to resolve after 6 wk, whereas footpads of the control animals progressed to ulceration and necrosis. Although disease did progress, albeit more slowly, in some of the treated mice, 85% were com-

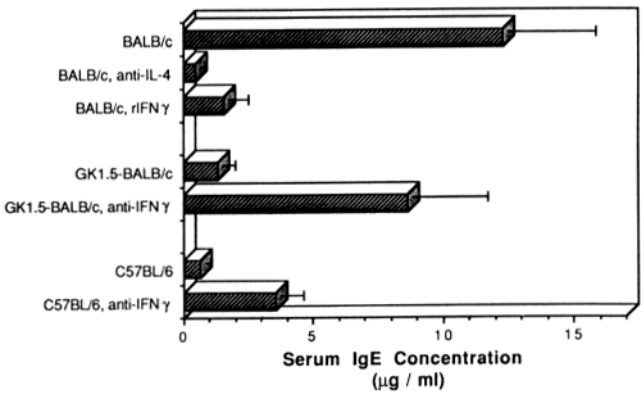

FIGURE 3. Effect of anti-IL-4, rIFN-γ, or anti-IFN-γ on the production of serum IgE after *L. major* infection. Values represent the mean ± SEM from five mice.

pletely healed by 8 wk. These data represent the compiled results from 48 mice treated with anti-IL-4. Histologic analyses of the footpads from the treated BALB/c mice confirmed that the resolution of swelling was associated with the disappearance of parasites (Fig. 2, *A* and *B*). In additional experiments, the coadministration of rIFN-γ (20 μg every 48 h) with anti-IL-4 therapy did not augment the beneficial effect achieved by anti-IL-4 alone (data not shown). Serum IgE levels were suppressed by >90% in anti-IL-4-treated mice, as compared with control-infected BALB/c mice (Fig. 3). In contrast, BALB/c mice that received weekly injections of either nonimmune rat sera or isotype-matched R4 6A2 anti-IFN-γ (rat IgG1) developed disease and elevated serum IgE levels indistinguishable from those in untreated infected BALB/c mice (data not shown).

Anti-IL-4 Effect Is Dependent on T Cells. When healed, anti-IL-4-treated BALB/c mice were rechallenged with viable promastigotes, they remained immune to reinfection (data not shown). Pooled spleen and lymph node cells from anti-IL-4-treated BALB/c mice that had resolved the primary infection were transferred into irradiated BALB/c recipients before challenge with *L. major* promastigotes. These mice, but not mice receiving naive BALB/c spleen cells, displayed substantial protection against the parasite, as assessed both by footpad swelling and ulceration (Fig. 4).

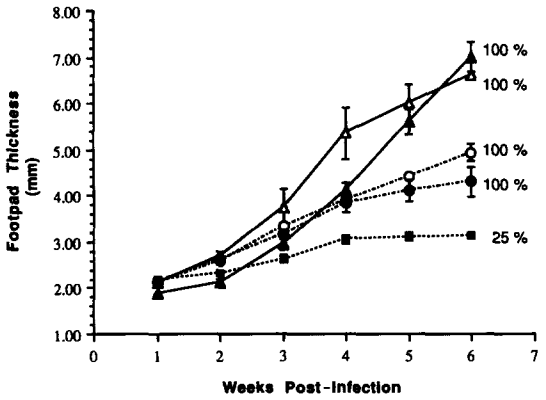

FIGURE 4. Adoptive transfer of anti-IL-4-induced immunity. Footpad measurements from untreated *L. major*-infected BALB/c mice (△) and *L. major*-infected 200-rad irradiated BALB/c mice that had been injected with 10^7 naive BALB/c spleen cells (▲), 10^7 pooled lymphoid cells from anti-IL-4-healed BALB/c (■), 10^7 CD4-depleted pooled lymphoid cells from anti-IL-4-healed BALB/c (○), or 10^7 CD8-depleted pooled lymphoid cells from anti-IL-4-healed BALB/c (●). Values represent the mean ± SEM from five mice. Percentage of footpads ulcerated at 6 wk is indicated.

Prior irradiation at these doses (in contrast to higher doses [9]) had no effect on the subsequent course of leishmaniasis as compared with nonirradiated controls. Treatment of the pooled cells with either anti-CD4 or anti-CD8 plus complement significantly abrogated the capacity of the transferred cells to prevent footpad swelling and completely reversed the prevention of ulceration. To establish further the requirement for T cells in the efficacy of anti-IL-4, athymic BALB/c *nu/nu* mice, which are unable to control leishmaniasis in the absence of T cell reconstitution (31), were infected with *L. major*. Such mice showed no alteration of disease progression in response to weekly anti-IL-4 injections (Fig. 5). Although footpad swelling was delayed in nude mice as compared with normal infected BALB/c mice, as previously reported (31), histologic analysis of the infected footpads and lymph nodes at week 6 revealed extensive parasitization of macrophages from both control and anti-IL-4-treated nude mice (data not shown).

Effect of Anti-IL-4 on IFN-γ and IL-4 mRNA. Prior studies have documented the correlation between the presence of IFN-γ mRNA and the capacity to heal *L. major* infection (1, 2). To assess the possible role of IFN-γ in the therapeutic response of anti-IL-4-treated mice, mRNA extracted from the spleens and lymph nodes of infected mice was examined for IFN-γ-specific transcripts. Although Northern blot analysis of splenic IFN-γ mRNA from the anti-IL-4-healed BALB/c mice revealed no differences as compared with untreated mice (Fig. 6, lanes *A* and *B*), message levels in the draining lymph nodes were approximately fourfold enhanced (Fig. 6, lanes *C* and *D*). Analysis of IL-4 mRNA in the same tissues similarly revealed no change in splenic message levels accompanying anti-IL-4 treatment, but a reciprocal fourfold decrease in specific mRNA in lymph node mRNA.

IFN-γ Does not Mediate Healing Induced by Anti-IL-4. To investigate further the mechanism of healing, in vivo neutralization of IFN-γ with anti-IFN-γ mAb, R4 6A2, was used to assess the role of this cytokine in anti-IL-4-treated BALB/c, C57BL/6, and GK1.5-BALB/c mice.

L. major-infected BALB/c mice receiving both anti-IL-4 and anti-IFN-γ antibodies contained the infection, as well as those receiving anti-IL-4 alone (Fig. 7 *A*). In marked contrast, infected C57BL/6 mice receiving anti-IFN-γ demonstrated progressive swelling of the footpads with ulceration by week 5 (Fig. 7 *B*). A correlation between

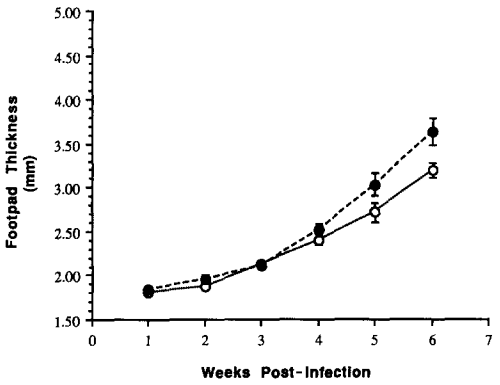

FIGURE 5. Effect of anti-IL-4 treatment on the progression of *L. major* infection in athymic BALB/c *nu/nu* mice. Footpad measurements for untreated (○) and anti-IL-4-treated (●) mice represent the mean ± SEM from four mice.

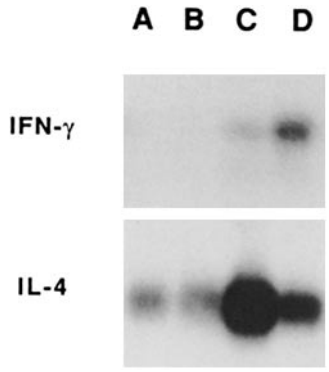

FIGURE 6. Effect of anti-IL-4 treatment on cytokine mRNA production during leishmaniasis. Poly(A)$^+$ RNA (10 μg) was isolated from the spleens of untreated or anti-IL-4-treated BALB/c mice (lanes *A* and *B*, respectively) or the popliteal lymph nodes of untreated or anti-IL-4-treated BALB/c mice (lanes *C* and *D*, respectively) 6 wk after infection with *L. major*. Northern hybridizations were performed using IL-4- and IFN-γ-specific probes.

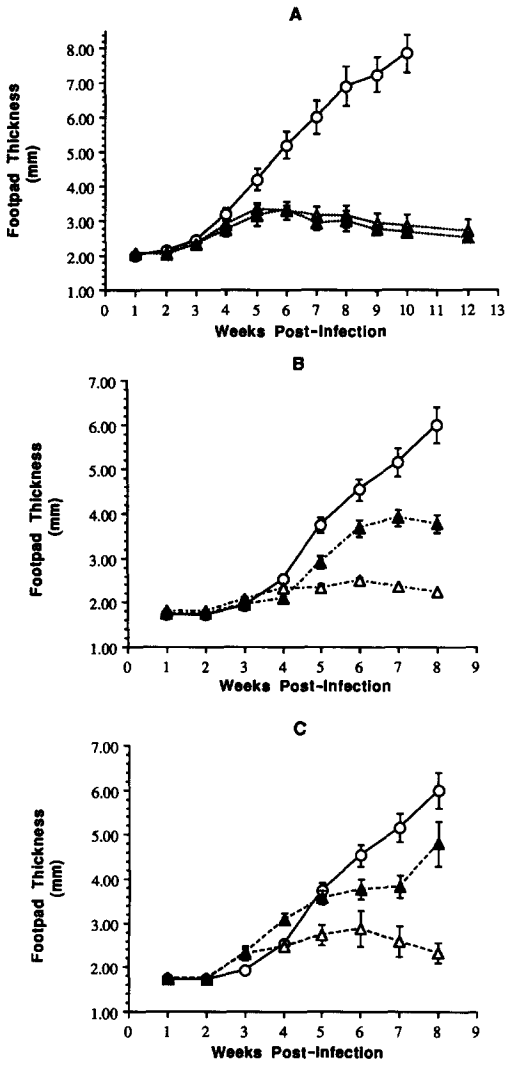

FIGURE 7. Effect of anti-IFN-γ on the progression of *L. major* infection. (*A*) Untreated BALB/c mice (○); anti-IL-4-treated (△); and anti-IL-4 + anti-IFN-γ-treated (▲) BALB/c mice. Footpad measurements represent the mean ± SEM from five mice. (*B*) Untreated BALB/c mice (○); C57BL/6 untreated (△); and anti-IFN-γ-treated (▲) mice. Footpad measurements represent the mean ± SEM from four mice. (*C*) Untreated BALB/c mice (○); GK1.5-BALB/c untreated (△); and anti-IFN-γ-treated (▲) mice. Footpad measurements represent the mean ± SEM from four mice.

footpad swelling and the presence of numerous parasites was confirmed by histologic examination of the footpads in which amastigote-parasitized macrophages were readily detectable (Fig. 8 *B*). Untreated C57BL/6 mice responded to infection with footpad swelling through the first 5–6 wk, followed by resolution without ulceration (Fig. 7 *B*). Resolution of disease was confirmed by histologic examination (Fig. 8 *A*). Similar results were seen when GK1.5-BALB/c mice were treated with anti-IFN-γ. Neutralization of IFN-γ resulted in progressive infection as assessed both by size of the local lesions and increasing numbers of amastigote-infected macrophages in the footpads (Figs. 7 *C* and 8, *C* and *D*, respectively). In both C57BL/6 and GK1.5-BALB/c mice, neutralization of IFN-γ resulted in significant increases in serum IgE levels as compared with untreated infected mice (Fig. 3).

Discussion

These studies demonstrate the profound effect of neutralization of IL-4 on the course of otherwise fatal infection by *L. major* in susceptible BALB/c mice. All treated mice had attenuated disease, and 85% were cured. The efficacy of anti-IL-4 treatment required the presence of T cells, and anti-IL-4-induced protection could be adoptively transferred in a manner dependent on both $CD4^+$ and $CD8^+$ cells. Surprisingly, there was no evidence that cure was mediated by the generation of IFN-γ, despite the demonstration that this cytokine was critical for healing in C57BL/6 or GK1.5-BALB/c mice. Further, treatment with rIFN-γ alone was insufficient to affect the course of disease in infected BALB/c mice, a finding consistent with at least two mechanisms for establishing control of leishmaniasis.

The effect of anti-IL-4 therapy was established by measurement of footpad thickness, histologic examinations demonstrating absence of parasites, and resistance of mice to rechallenge with promastigotes 12 wk subsequent to the initial infection. Neither control rat sera nor an isotype-matched mAb had any effect on the course of disease, suggesting that neutralization of IL-4 is required for activity. The finding that anti-IL-4 therapy abrogated the rise in serum IgE seen in untreated, infected BALB/c mice further documents the neutralization of IL-4 in vivo (32–34). Treatment of infected *nu/nu* BALB/c mice with anti-IL-4 had no effect on the course of disease, indicating that T cells are required. The latter experiment further excluded the possibility of direct antileishmanial activity by the anti-IL-4 mAb.

Because IL-4 has been proposed as a requisite growth factor for Th2 cells (13, 35), our initial assumption was that anti-IL-4 therapy was effective due to a selective blockade of the expansion of *Leishmania* antigen-specific Th2 cells. The decrease in IL-4 mRNA and reciprocal increase in IFN-γ mRNA that occurred in the lymph node cells draining the lesion were consistent with a proportionately greater expansion of Th1 cells in response to inhibition of Th2 cell division by anti-IL-4. However, several observations suggest that this may not be the only mechanism. rIFN-γ has also been shown to effectively block Th2 cell proliferation in vitro (36), but despite administration of rIFN-γ at doses shown to effectively ameliorate fatal infection by *T. gondii* (29, 30) or by *T. cruzi* (31), mice infected with *L. major* developed progressive infection. Suppression of serum IgE, comparable with that seen in anti-IL-4-treated mice, demonstrated that the administered rIFN-γ induced systemic effects up to 5 wk after infection. As macrophages are the only host cell parasitized by *Leishmania*, neutralization of the capacity of IL-4 to deactivate macrophages, even in the pres-

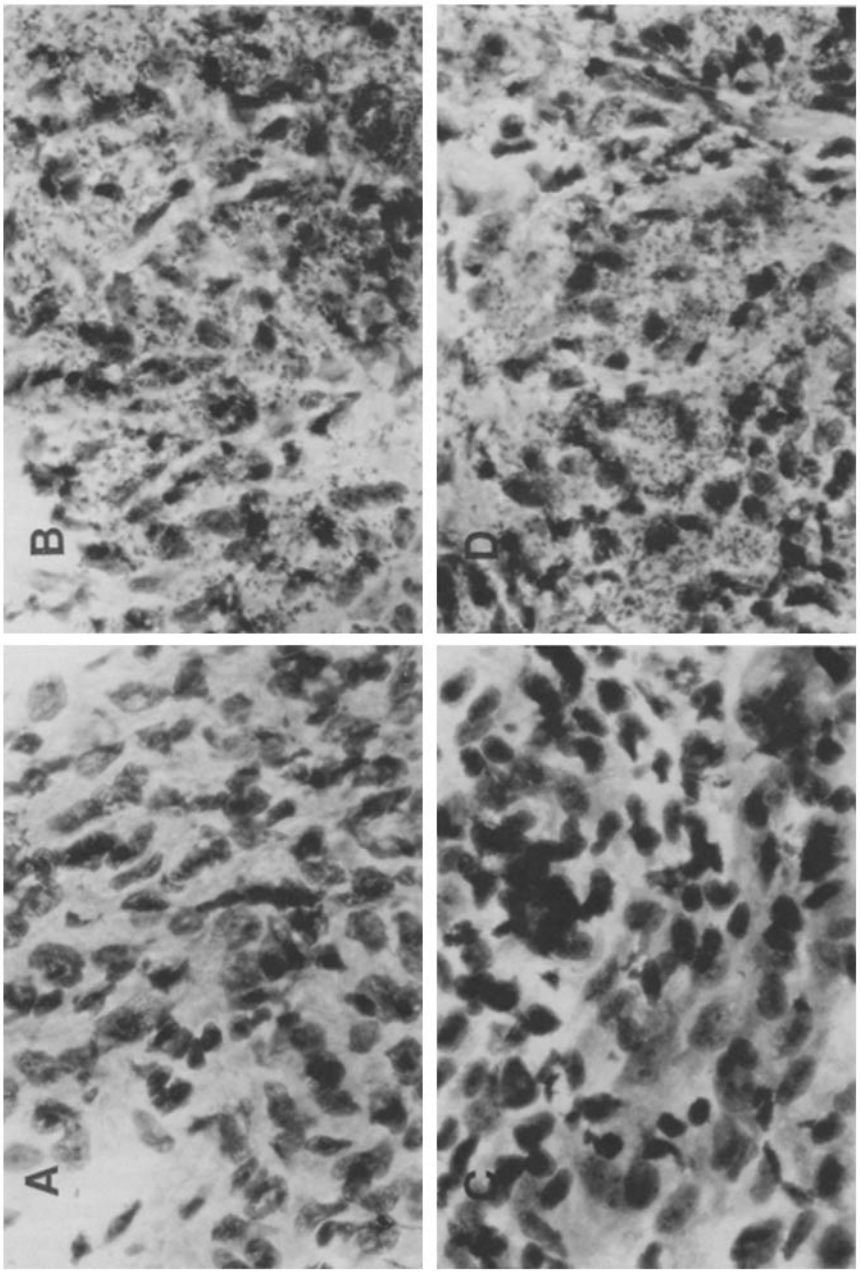

FIGURE 8. Effect of anti-IFN-γ on the progression of *L. major* infection in C57BL/6 and GK1.5-BALB/c mice. Tissue sections were prepared from footpads from 8-wk-infected untreated (*A*) or anti-IFN-γ-treated (*B*) C57BL/6 mice and from untreated (*C*) or anti-IFN-γ-treated (*D*) GK1.5-BALB/c mice (×750).

ence of IFN-γ (37, 38), may be an important mechanism underlying the efficacy of anti-IL-4 therapy.

Prior studies of healer C57BL/6 or GK1.5-BALB/c mice established a correlation between production of IFN-γ in response to *Leishmania* antigen stimulation in vitro (1) and the presence of IFN-γ mRNA in lymph node and spleen cells in vivo (2) with resolution of infection. The identification of IFN-γ as a major macrophage-activating factor and the requirement for macrophage activation to kill this intracellular parasite have been well established (5, 7, 30, 39). The studies reported here demonstrate that IFN-γ is required for healing in these mice, since the administration of neutralizing anti-IFN-γ antibody resulted in progressive, uncontrolled infection both in C57BL/6- and anti-CD4-pretreated BALB/c mice. Similar findings have been reported recently in studies of *L. major* infection in C3H/HeN mice (40). Despite the requirement for IFN-γ in resolution of disease in these mice, rIFN-γ did not protect BALB/c mice by itself. It did not enhance the therapeutic effects of anti-IL-4 either, suggesting that while important to the outcome of disease, IFN-γ is not sufficient by itself for the resolution of leishmaniasis in BALB/c mice.

Importantly, cure mediated by neutralization of IL-4 appeared independent of IFN-γ, since cotreatment of these mice with neutralizing anti-IFN-γ mAb did not abrogate the therapeutic effect of the anti-IL-4. Treatment with anti-IL-4 established protective cellular immunity, as assessed by cell transfer experiments, that resided in both the $CD4^+$ and $CD8^+$ populations. Although CD4 depletion was slightly more effective than CD8 depletion in reversing the attenuation of footpad swelling, the CD8 requirement supports recent observations establishing a protective role for these cells in leishmaniasis (41, 42). The possibility that macrophage-activating factors other than IFN-γ (43, 44) or contact-mediated macrophage activation (45, 46) are involved in the mechanism underlying the potent effects of anti-IL-4 therapy of leishmaniasis remains to be established. The elucidation of the mechanisms involved should uncover novel redundant systems for the control of intracellular pathogens.

Summary

BALB/c mice infected with *Leishmania major* develop fatal, progressive disease, despite an immune response characterized by expansion of $CD4^+$ T cells in the draining lymph nodes. The immune response has been further characterized by a lack of IFN-γ mRNA, but increased IL-4 mRNA in lymphoid tissues, and striking elevation of serum IgE. Treatment of infected BALB/c mice with rIFN-γ at doses shown to be beneficial in other protozoan infections was insufficient to ameliorate *L. major* infection. In contrast, neutralization of IL-4 by six weekly injections of mAb 11B11 led to attenuation of disease in 100% of animals, and complete cure in 85%. Resolution of disease required the presence of T cells, and recovered mice remained resistant to reinfection at 12 wk. This immunity was adoptively transferable and was dependent on both $CD4^+$ and $CD8^+$ cells. Although administration of anti-IL-4 was associated with fourfold increase in IFN-γ mRNA in lymph node cells draining the lesion, the coadministration of neutralizing R4 6A2 anti-IFN-γ mAb had no effect on resistance to disease. This was in marked contrast to resolution of disease in both resistant C57BL/6- and GK1.5-pretreated BALB/c mice that was abrogated by in vivo treatment with anti-IFN-γ. These data suggest a novel mechanism of cel-

lular immunity established by interference with the development of Th2 cells during infection.

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